Synthesis of the posterior determinant Nanos is spatially restricted by a novel cotranslational regulatory mechanism

Ira E. Clark, David Wyckoff and Elizabeth R. Gavis

Nanos (Nos) protein is required in the posterior of the *Drosophila* embryo to promote abdominal development, but must be excluded from the anterior to permit head and thorax development [1,2]. Spatial restriction of Nos is accomplished by selective translation of the 4% of *nos* mRNA localized to the posterior pole and translational repression of the remaining unlocalized mRNA [3–5]. Repression is mediated by a 90-nucleotide translational control element (TCE) in the *nos* 3′ untranslated region (UTR) and the TCE-binding protein Smaug [4,6,7], but the molecular mechanism is unknown. We used sucrose density gradient sedimentation to ascertain whether unlocalized *nos* mRNA is excluded from polysomes and therefore repressed during translational initiation. Surprisingly, a significant percentage of *nos* mRNA was found to be associated with polysomes, even in mutants in which all *nos* mRNA is unlocalized and repressed. Using a regulated *Drosophila* cell-free translation system, we showed that ribosomes contained within these polysomes are capable of elongation *in vitro*, under conditions in which synthesis of Nos protein is repressed. Thus, synthesis of ectopic Nos protein is inhibited by a novel regulatory mechanism that does not involve a stable arrest of the translation cycle.

Address: Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA.

Correspondence: Elizabeth R. Gavis
E-mail: lgavis@molbio.princeton.edu

Received: **17 July 2000**
Revised: **23 August 2000**
Accepted: **23 August 2000**

Published: **6 October 2000**

Current Biology 2000, 10:1311–1314

0960-9822/00/$ – see front matter

Results and discussion

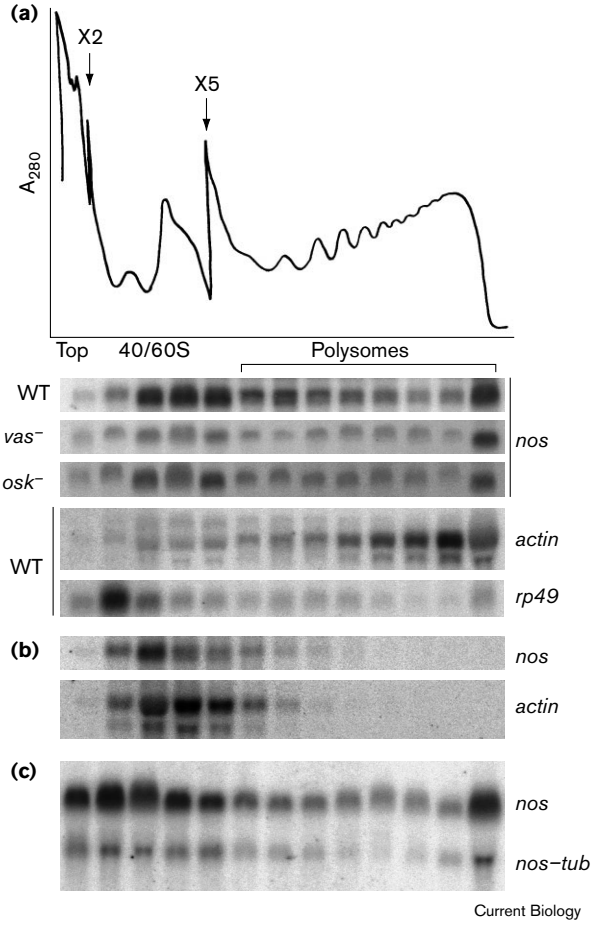
Extracts of preblastoderm embryos were fractionated by sucrose density centrifugation to separate polysomes from ribosomal subunits and mRNPs. The relative abundance of *nos* mRNA in each fraction was assessed by northern blot analysis. Under standard conditions, well-translated mRNAs such as *actin* are highly enriched in fractions containing polysomes, whereas *rp49*, which is repressed at initiation in early embryos [8], is effectively excluded from these fractions (Figure 1a). Surprisingly, 53% of *nos* mRNA in early embryonic extracts comigrated with polysomes (Figure 1a). This fraction far exceeds the 4% of *nos* mRNA that is localized and actively translated, and therefore suggests that repressed *nos* mRNA is associated with polysomes. When the extract was treated with EDTA to remove Mg^{2+} and destabilize polysomes, both *actin* and *nos* mRNAs sedimented more slowly in the sucrose gradient (Figure 1b). Moreover, when the antibiotic puromycin was used to specifically disrupt polysomes [9], these mRNAs were similarly shifted to the more slowly sedimenting monosomal fractions of the gradient (Figure 2).

To confirm that *nos* transcripts cosedimenting with polysomes do not represent the localized subset of *nos* mRNA, we performed an analogous analysis of *nos* mRNA in extracts of embryos from *vasa*$^-$ (*vas*$^-$) and *oskar*$^-$ (*osk*$^-$) females. Although present at wild-type levels, all *nos* mRNA in these embryos is unlocalized and translationally repressed [3]. Nevertheless, 63% and 50% of *nos* mRNA from *vas*$^-$ and *osk*$^-$ extracts, respectively, cofractionated with polysomes (Figure 1a). As in the case of wild-type extracts, puromycin treatment of *vas*$^-$ extracts resulted in a shift of *nos* mRNA from polysomal to monosomal fractions of the gradient (data not shown).

Although ~50% of *nos* mRNA was engaged with polysomes, a large portion of the mRNA was not polysomal (Figure 1a). This nonpolysomal fraction may reflect inefficient translation of *nos* relative to transcripts such as *actin*, possibly as a result of secondary structure within the *nos* 5′UTR (I.E.C. and E.R.G., unpublished observations). Alternatively, it may be due to the regulatory action of the TCE. To test the latter hypothesis, we compared the sedimentation of repressed *nos* mRNA with that of a chimeric *nos–tub3′UTR* mRNA, in which the *nos* 3′UTR is replaced by the *α-tubulin* 3′UTR and is thus unlocalized and unregulated [3]. Sedimentation of the unregulated *nos–tub3′UTR* mRNA was comparable to that of endogenous wild-type *nos* mRNA (Figure 1c), indicating that the *nos* 3′UTR affects neither the degree of polysome association nor the size of polysomes associated with *nos* mRNA. While we cannot exclude the possibility that *nos* mRNA is associated with a large nonpolysomal particle that is sensitive to puromycin, EDTA and cycloheximide (see below), the most direct interpretation of our data is that repressed, unlocalized *nos* mRNA is engaged with actively translating ribosomes.

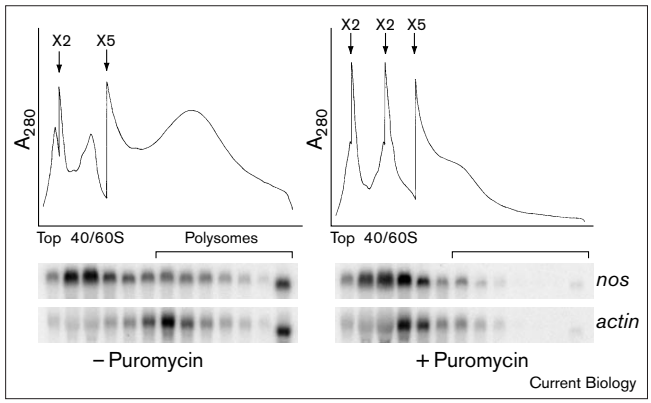
To date, the best model for regulation of translation by 3′UTR sequences involves modulation of synergistic

Figure 1



Translationally repressed *nos* mRNA is associated with polysomes. **(a)** Extracts of 0–2 h embryos from wild-type (WT), *vas*$^{-}$ (*vas*^{PD}/*vas*^{D1}) or *osk*$^{-}$ (*osk*^{54}) females were fractionated through 20–45% sucrose gradients and subjected to northern blot analysis. A typical ultraviolet (UV) absorbance profile is shown at the top with scale changes indicated. Northern blots were probed for *nos*, *actin* and *rp49*. The rightmost lane of each gel contains a volume equivalent of the solubilized gradient pellet. EDTA and puromycin disruption experiments suggest that the pellet contains very large polysomes (see below and Figure 2). Phosphorimaging was used to quantitate the percentage of each transcript present in polysomal fractions (bracketed) from wild-type, *vas*$^{-}$ and *osk*$^{-}$ extracts, respectively, as follows: *nos* (53%, 63%, 50%); *actin* (88%, 85%, 84%); *rp49* (20%, 23%, 22%). **(b)** Extract of wild-type 0–2 h embryos fractionated over a 20–45% sucrose gradient in the presence of EDTA. Polysomes were completely disrupted, as assayed by UV absorbance (not shown) and *actin* mRNA sedimentation. *nos* mRNA was only found in slowly sedimenting fractions coincident with monosomes and ribosomal subunits. **(c)** Extract of 0–2 h embryos from wild-type females bearing the *nos–tub3′UTR* transgene was analyzed as described in (a). The unregulated *nos–tub3′UTR* mRNA (*nos–tub*) sedimented comparably to translationally regulated endogenous *nos* mRNA (34% of *nos–tub3′UTR* in polysomal fractions versus 31% of *nos* in this experiment).

Figure 2

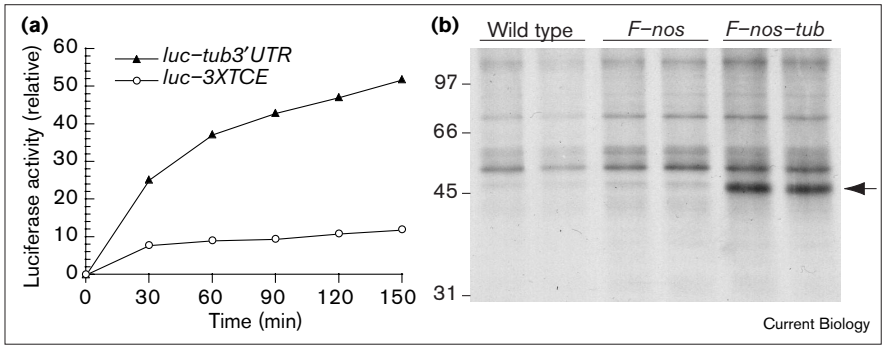


Rapid sedimentation of *nos* mRNA requires polysome integrity. Extracts of 0–2 h wild-type embryos were mock-treated or treated with puromycin to disrupt polysomes before sucrose gradient sedimentation. Because puromycin activity requires progression through an elongation cycle, polysome-stabilizing agents, such as high Mg^{2+} and the elongation inhibitor cycloheximide, were omitted. Omission of these components led to a general decrease in the ratio of large to small polysomes in the control. *nos* and *actin* mRNAs were found only in slower sedimenting fractions after puromycin treatment. Polysomal fractions (bracketed) in the absence of puromycin contained 32% and 70% of *nos* and *actin* mRNA, respectively. After puromycin treatment, analogous fractions (bracketed) contained only 6% and 11%, respectively, of these transcripts.

interactions between poly(A)-binding protein and factors binding to the cap, which in turn alters the efficiency of initiation [10]. Our data indicate that translational repression by the *nos* 3′UTR does not operate at the level of initiation, but rather at a more downstream event during elongation or termination. To investigate these phases of translation, we prepared cell-free, translationally active extracts of preblastoderm *Drosophila* embryos. These extracts, which are not nuclease-treated and therefore retain all endogenous mRNA, supported translation that was dependent on both the cap and poly(A) tail (see Supplementary material). Furthermore, the extracts were competent for TCE-mediated repression of exogenous luciferase reporter mRNAs. When compared with luciferase mRNA bearing the *α-tubulin* 3′UTR (*luc–tub3′UTR*), a luciferase mRNA bearing three tandem copies of the TCE (*luc–3×TCE*) yielded 5–7-fold lower levels of luciferase (Figure 3a). Similarly, *luc–nos+2* mRNA, which contains a single copy of the TCE and an adjacent 88 nucleotide region of the *nos* 3′UTR [11], was repressed 4–6-fold (data not shown).

Immunoprecipitation experiments indicated that these extracts were additionally capable of TCE-mediated repression of endogenous *nos* mRNA. For these experiments, we prepared translationally active extracts of embryos from females carrying either *F–nos* or *F–nos–tub3′UTR* transgenes. These transgenes are identical to the wild-type *nos* and *nos–tub3′UTR* transgenes, respectively, except that they encode a Nos protein with an amino-terminal FLAG epitope. *F–nos* and *F–nos–tub3′UTR* mRNAs are indistinguishable

Figure 3



TCE-dependent repression in a translationally active cell-free extract. **(a)** Translationally active cell-free extract from 0–2 h wild-type embryos (see Supplementary material) was programmed with synthetic luciferase reporter mRNAs bearing either the *α-tubulin* 3′UTR (*luc–tub3′UTR*) or three tandem copies of the *nos* TCE (*luc–3×TCE*). Luciferase levels shown were normalized to values obtained by translating equivalent amounts of mRNA in rabbit reticulocyte extracts, which are unregulated. Both reporter mRNAs were equally stable over the course of the reaction (data not shown). Smibert *et al.* [17] have shown that TCE-dependent repression of exogenous mRNAs in a similar cell-free extract requires the presence of Smaug protein. **(b)** Extracts of 0–2 h wild-type (lanes 1,2), *F–nos* (lanes 3,4) and *F–nos–tub3′UTR* (*F–nos–tub*, lanes 5,6) embryos were incubated for 45 min in the presence of [^{35}S]methionine to allow translation of endogenous mRNA. Volume equivalents of the reactions were immunoprecipitated with anti-FLAG antibody and subjected to SDS–PAGE and autoradiography. The arrow indicates the position of the F–Nos protein, which was only detectable in the *F–nos–tub3′UTR* samples. Molecular weights shown are in kDa.

from their untagged counterparts in both RNA localization and translational regulation (see Supplementary material).

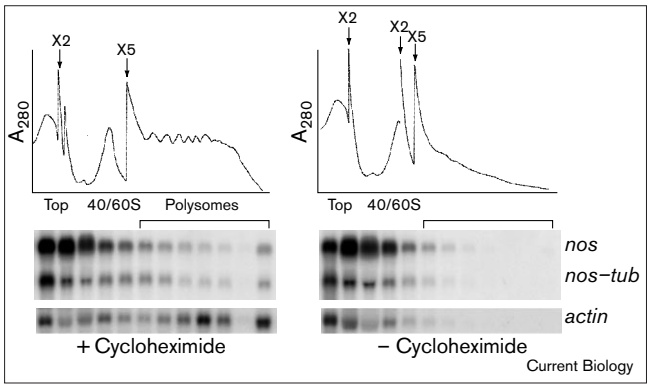
We allowed endogenous polysomes from *F–nos* and *F–nos–tub3′UTR* extracts to complete translation *in vitro* in the presence of [^{35}S]methionine, and used immunoprecipitation to monitor *de novo* synthesis of F–Nos protein. While radiolabeled F–Nos could be immunoprecipitated from *F–nos–tub3′UTR* extracts, it was undetectable from *F–nos* extracts (Figure 3b). Northern analysis of the transgenic lines used indicated that *F-nos* mRNA is 34% more abundant than *F-nos-tub3′UTR* mRNA (data not shown). These translation extracts are therefore not only capable of repressing exogenous reporter transcripts, but also recapitulate translational repression of endogenous unlocalized *nos* mRNA.

Attempts to incorporate radiolabeled synthetic mRNA into polysomes in these extracts indicated that translational initiation was inefficient (I.E.C., unpublished observations). This allowed us to perform translational runoff experiments to determine whether ribosomes bound to repressed, endogenous *nos* mRNA were arrested during elongation or termination. For these experiments, translationally active extract was prepared from *nos–tub3′UTR* embryos, which contain both repressed wild-type *nos* and unregulated *nos-tub3′UTR* mRNAs; similar results were obtained with wild-type extract (data not shown). Endogenous polysomes in the extract were allowed to complete translation *in vitro* in the absence of exogenous mRNA as above. As monitored by UV absorbance, bulk polysomes remained intact when these reactions included the elongation inhibitor cycloheximide, but were efficiently disassembled in its absence (Figure 4). The ability of cycloheximide to stabilize polysomes indicates that disassembly results as ribosomes complete translation and release from mRNA. Disassembly of bulk polysomes further indicates that initiation on endogenous mRNA is inefficient in these reactions. As confirmation, identical results were obtained in the presence of the initiation inhibitor pactamycin (data not shown). Taken together, these results show that the experiment monitors *bona fide* translational runoff.

While bulk polysomes are disassembled during translational runoff, polysomes on specific transcripts that are arrested during elongation or termination would be expected to remain intact even in the absence of cycloheximide. We measured translational runoff of specific endogenous mRNAs by comparing the amount of mRNA in polysomal fractions in the cycloheximide-arrested control to that present in equivalent fractions in the untreated translating sample. By this analysis, 96% of polysomal *actin* mRNA was subject to runoff. Surprisingly, we found that ~70% of polysomal *nos* mRNA was released after runoff, as compared with 78% of polysomal *nos–tub3′UTR* mRNA (Figure 4). Comparable results were obtained when pactamycin was included in the translation reaction to prevent new initiation (data not shown). Northern analysis indicated that total *nos* RNA levels were unchanged during the course of the translation reaction (data not shown). These observations demonstrate that ribosomes are capable of elongation and release from *nos* mRNA even under conditions in which endogenous Nos synthesis is undetectable. Thus, TCE-mediated repression does not impose a stable arrest of elongating or terminating ribosomes.

We propose two possible models for repression. First, factors bound to the TCE may degrade or destabilize the nascent polypeptide chain. While this mechanism may not strictly regulate the translation cycle, it should operate

Figure 4



Translationally repressed *nos* mRNA is subject to translational runoff *in vitro*. Extracts of 0–2 h *nos–tub3′UTR* embryos were incubated for 90 min to allow endogenous polysomes to complete translation and disassemble. A control reaction containing cycloheximide was processed in parallel. After incubation, reactions were fractionated by sucrose gradient sedimentation and analyzed as in Figure 1. *nos* and *actin* mRNAs were shifted to slower sedimenting fractions after translational runoff. Percentages of mRNA found in polysomal fractions (bracketed) in the presence or absence of cycloheximide, respectively, were: *nos* (7%, 2%); *nos–tub3′UTR* (12%, 3%); *actin* (60%, 3%). Aliquots of the translation extract were also programmed in parallel with *luc–tub3′UTR* or *luc–3×TCE* reporter RNA; fourfold repression of exogenous RNA was observed in this experiment.

cotranslationally, as the TCE works only in *cis*. Alternatively, the TCE and its associated factors may alter the processivity of the ribosome and promote premature release of either the ribosome or nascent polypeptide, followed by degradation of the incomplete protein product. Polysome association of translationally repressed transcripts has also been observed for the heterochronic genes *lin-14* and *lin-28* in *Caenorhabditis elegans* ([12]; K. Seggerson and E. Moss, personal communication). While elongation has not yet been examined for these mRNAs, it is tempting to speculate that they may be regulated by a mechanism similar to that used for *nos*. Intriguingly, the *cis*-regulatory element for each of these mRNAs lies within the 3′UTR and has the capacity to form a double-stranded structure [13,14].

Temporal considerations of *nos* expression and function underscore the advantages of regulating translation at a step after initiation. *nos* mRNA is actively translated in nurse cells before its deposition in the oocyte [15]. Post-initiation mechanisms may be particularly effective at rapidly inactivating mRNAs, such as *nos*, that are already engaged with ribosomes. Furthermore, repressing mRNA after initiation may allow for rapid activation of silenced mRNAs. Nos protein promotes abdominal development by repressing translation of maternal *hunchback* (*hb*) mRNA, which is activated at fertilization [16]. Derepression of *nos* mRNA that has been preloaded with ribosomes may allow Nos to be rapidly synthesized at the posterior pole upon localization, before activation of *hb*. Indeed, post-initiation mechanisms of translational control may prove to be a powerful mechanism for enhancing spatial and temporal fidelity of gene expression.

Supplementary material

Supplementary material including additional methodological detail and data demonstrating dependence of the extracts on the cap and poly(A) tail is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank D. Chagnovich and R. Lehmann for reagents; K. Seggerson and E. Moss for communicating results prior to publication; S. Crucs, G. Deshpande, T. Huang and P. Schedl for comments on the manuscript; and members of the Gavis lab and A. Sachs for helpful comments during the course of this work. I.E.C. was supported by NIH Training Grant (CA09528) and NIH NRSA (F32 GM18770-01A1). This work was supported by the NSF (IBN95-06828) and a Beckman Young Investigator Award to E.R.G.

References

1. Wang C, Lehmann R: **Nanos is the localized posterior determinant in *Drosophila*.** *Cell* 1991, **66:**637-647.
2. Gavis ER, Lehmann R: **Localization of *nanos* RNA controls embryonic polarity.** *Cell* 1992, **71:**301-313.
3. Gavis ER, Lehmann R: **Translational regulation of *nanos* by RNA localization.** *Nature* 1994, **369:**315-318.
4. Gavis ER, Lunsford L, Bergsten SE, Lehmann R: **A conserved 90 nucleotide element mediates translational repression of *nanos* RNA.** *Development* 1996, **122:**2791-2800.
5. Bergsten SE, Gavis ER: **Role for mRNA localization in translational activation but not spatial restriction of *nanos* RNA.** *Development* 1999, **126:**659-669.
6. Smibert CA, Wilson JE, Kerr K, Macdonald PM: **smaug protein represses translation of unlocalized *nanos* mRNA in the *Drosophila* embryo.** *Genes Dev* 1996, **10:**2600-2609.
7. Dahanukar A, Walker JA, Wharton RP: **Smaug, a novel RNA-binding protein that operates a translational switch in *Drosophila*.** *Mol Cell* 1999, **4:**209-218.
8. Al-Atia GR, Fruscoloni P, Jacobs-Lorena M: **Translational regulation of mRNAs for ribosomal proteins during early *Drosophila* development.** *Biochemistry* 1985, **24:**5798-5803.
9. Blobel G, Sabatini D: **Dissociation of mammalian polyribosomes into subunits by puromycin.** *Proc Natl Acad Sci USA* 1971, **68:**390-394.
10. Sachs AB, Sarnow P, Hentze MW: **Starting at the beginning, middle and end: translation initiation in eukaryotes.** *Cell* 1997, **89:**831-838.
11. Gavis ER, Curtis D, Lehmann R: **Identification of *cis*-acting sequences that control *nanos* RNA localization.** *Dev Biol* 1996, **176:**36-50.
12. Olsen PH, Ambros V: **The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation.** *Dev Biol* 1999, **216:**671-680.
13. Moss EG: **Non-coding RNAs: Lightning strikes twice.** *Curr Biol* 2000, **10:**R436-439.
14. Crucs S, Chatterjee S, Gavis ER: **Overlapping but distinct RNA elements control repression and activation of *nanos* translation.** *Mol Cell* 2000, **5:**457-467.
15. Wang C, Dickinson LK, Lehmann R: **Genetics of *nanos* localization in *Drosophila*.** *Dev Dynam* 1994, **199:**103-115.
16. Wickens M, Kimble J, Strickland S: **Translational control of developmental decisions.** In *Translational Control.* Edited by Hershey JWB, Mathews MB, Sonenberg N. Plainview: Cold Spring Harbor Laboratory Press; 1996:411-450.
17. Smibert CA, Lie YS, Shillinglaw W, Henzel WJ, Macdonald PM: **Smaug, a novel and conserved protein, contributes to repression of *nanos* mRNA translation *in vitro*.** *RNA* 1999, **5:**1535-1547.